AD_____

Award Number: DAMD17-00-1-0426

TITLE: The Role of ROS in Breast Cancer Metastasis

PRINCIPAL INVESTIGATOR: Ulla G. Knaus, Ph.D.

CONTRACTING ORGANIZATION: The Scripps Research Institute
La Jolla, California 92037

REPORT DATE: May 2001

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	May 2001	Annual (1 May 00 - 30 Apr 01)

4. TITLE AND SUBTITLE
The Role of ROS in Breast Cancer Metastasis

5. FUNDING NUMBERS
DAMD17-00-1-0426

6. AUTHOR(S)
Ulla G. Knaus, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The Scripps Research Institute
La Jolla, California 92037

E-Mail: uknaus@scripps.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

Numerous cancer cells generate reactive oxygen species (ROS), which are thought to promote cell proliferation, cell motility and invasion, prerequisites for tumor metastasis. Recently novel ROS-generating enzymes termed Nox have been identified in epithelial cells. Transfer of Nox into non-transforming epithelial cells increased ROS production and rendered these cells tumorigenic. Our project will identify Nox family members in cancer cells and evaluate if they are required for constitutive ROS generation and altered cell behavior.

Breast cancer cell lines were screened by RT-PCR for the presence of identified *nox* genes and did not contain known Nox family members, as expected due to their tissue specificity. The regulation of Nox-based enzyme systems might be under control of small GTPases and their effectors. We will test this hypothesis by introducing mutants of these regulatory molecules into breast cancer cells via adenoviral transfer and evaluate ROS generation. To test the involvement of Nox family members in cell migration and invasion, in vitro assays systems are used to test Nox-transfected cell lines and cancer cell lines, which are inhibited in their ability to generate ROS. The activity of deregulated Nox proteins leading to ROS generation may have wide ranging implications in tumorigenic events including metastasis.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Reactive oxygen species, cell migration, invasion, signal transduction	6
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

INTRODUCTION:

Certain human carcinoma cell lines produce reactive oxygen species (ROS) constitutively. This affords them potentially with an advantage in cell proliferation, and migration and invasion of surrounding tissues by degrading the surrounding extracellular matrix and by increasing their motility. The basis for generation or upregulation of ROS in cancer cells is so far unknown. We hypothesized that recently identified, novel ROS-producing enzyme systems might be involved and proposed to define members of this family in breast cancer cells and to evaluate their role in cell migration.

BODY:

Characterization of the ROS generating enzyme in cancer cells

We have spent considerable time and effort in analyzing the identity of the ROS-generating enzyme in cancer cells. According to our hypothesis, we focused our efforts on NADPH oxidase components and cytochrome b $_{558}$ homologs. Initially, we probed in collaboration with Dr. Lambeth for the presence of the first characterized gp91^{phox} homolog Nox1 using RNA derived from normal breast epithelial and breast cancer cell lines. Since the data obtained by Northern blotting with *nox1* were inconclusive and novel members of the Nox family were identified, we decided to determine the presence of these *nox* genes in ROS-generating cancer cells by RT-PCR (1-5). Using *nox*-positive cells as control we designed primers for *nox1*, *nox4* and *nox3*. We detected full-length *nox1* in the colon cancer cell line CaCo2, but not in several different breast cancer cell lines. Similarly, *nox4* was present in HEK293 cells but not in the breast cancer cells tested. At this time we are still working on the conditions for *nox3* PCR. Nox3 was reported to be expressed in fetal liver tissue and Hep2G cells. Since we have no easily available source for human fetal liver DNA, Hep2G cells are currently used for RT-PCR. Additionally, we designed primers for *nox2* (formerly gp91^{phox}). While neutrophil control RNA showed Nox2 as expected, this gene was not detected in the tested breast cancer cells.

We conclude from these first experiments that breast cancer cells do not contain the NADPH oxidase component Nox2 and might produce ROS using other Nox family members. Since the narrow range of tissue distribution of the novel genes Nox1, Nox3 and Nox4 seems to indicate Nox family member tissue specificity, we searched the Est database for additional homologs. Two potential Nox sequences were found and their presence in breast cancer cell lines will be tested.

Identification of signals regulating ROS production in cancer cells

To investigate which signaling pathways might enable cancer cells to produce ROS constitutively, dominant negative and positive signaling molecules have to be introduced into these cells. Most breast cancer cells are relatively difficult to transfect with high efficiency using lipid-mediated gene transfer. We decided to establish an adenovirus-based infection system, which would allow infecting 95-100% of the cells without high toxicity. Three different control viruses encoding the green fluorescent protein GFP were produced. We used Fiber5 or Fiber3 containing adenovirus, choosing either recombination in HEK293 or in E. coli. After infection of breast cancer cells at different conditions and virus concentrations, transfection efficiency (i.e. production of GFP) was determined by FACS analysis. In light of our results, we decided to

establish an adenovirus Fiber5 system using *E. coli* for recombination followed by virus propagation in HEK293 cells.

Several signaling molecules have been implicated in superoxide-generating enzyme systems in neutrophils or fibroblasts. Predominantly the small GTPases Ras and Rac seem to regulate aspects of ROS production in these cells. Our own work in neutrophils shows an important role for Rac and its effector kinase Pak in ROS generation. We have started to clone several of these signaling molecules in wildtype, constitutively active and dominant negative form into the adenoviral vector using short N-terminal tags as expression markers.

Evaluation of ROS as second messengers in cell movement of cancer cells

Our first step was a more thorough analysis of certain potential ROS inhibitors in selected cancer cell lines. The flavoprotein inhibitor DPI inhibited ROS production dose-dependently. Additionally, several inhibitors of the mitochondrial respiratory chain were tested and did not affect ROS release. A very specific inhibitor of Cox-2 (Ns-398) and the intracellular Ca^{2+} antagonist TMB-8 were used and proved ineffective. Since our goal is the evaluation of cancer cell migratory behavior due to ROS generation, we coated Boyden chamber inserts with serum, fibronectin, vitronectin, collagen and BSA and assessed the extent of cancer cell migration. While there were slight differences in attachment, once the cells were attached all surfaces (except of BSA our control surface) were similar in promoting undirected migration. We will use fibronectin and collagen in our experiments. Interestingly, ROS production was not inhibited by detachment for 12 h, which was created by coating of cell culture dishes with polymethacrylate. Starvation of cancer cells for 24-48 h suppressed ROS generation, which might suggest an autocrine loop or feedback mechanism. This observation will be pursued in the future by investigating potential regulatory growth factors or hormones.

Since the identity of the ROS-generating enzyme in breast cancer cells is still under investigation, we decided to evaluate at this time the effect of stably transfected Nox on cell migration. We obtained Nox4 overexpressing HEK293 cells and vector control cells through collaboration. Initial assays show a certain phenotype change by Nox4 (cancer cell-specific piling up of cells on top of each other). Using the homovanillic acid assay, which measures production and release of hydrogen peroxide, we detected production of ROS in Nox4 expressing cells but not in vector expressing cells. Nox4-HEK293 cells do not attach very well and our next experiments are targeted to find a coating surface, which allows sufficient attachment to compare Nox4 and vector cells in their migratory behavior. We have also cloned Nox1 by RT-PCR, which could also be used to generate stable cell lines for our investigations.

KEY RESEARCH ACCOMPLISHMENTS:

- RNA isolation and RT-PCR from breast epithelial, breast cancer cells and control cells to detect *nox* genes.
- Establishment of an adenovirus Fiber5 system and determination of its efficiency in breast cancer cells.
- Assessment of parameters for ROS generation and cell migration of cancer cells. Analysis of a stably transfected Nox4-containing cell line and the vector control cell line.

REPORTABLE OUTCOMES:

This award just started and additional work has to be accomplished prior to publication or presentation at meetings.

CONCLUSIONS:

Two different Nox family members have been reported since our proposal was submitted and funded. Their functions and regulation are still unknown, but are implicated in promoting cell growth and tumor formation in mice. The distribution of Nox family members seems to be tissue specific and no isoform has been identified to date in breast epithelium. Identification and characterization of additional Nox proteins will shed light on their regulation in cancer cells. ROS have been implicated in tumor metastasis in mice and the deregulated or altered state of Nox proteins in cancer cells might mediate these effects.

REFERENCES:

1. Suh, Y.-A. et al. Cell transformation by the superoxide-generating oxidase Mox1. *Nature* 401:79, 1999.
2. Dupuy, C., Ohayon, R., Valent, A., Noel-Hudson, M.-S., Deme, D., and Virion, A. Purification of a Novel Flavoprotein Involved in the Thyroid NADPH Oxidase. *J. Biol. Chem.* 274:37265, 1999.
3. Banfi, B., et al. A Mammalian H^+ Channel Generated Through Alternative Splicing of the NADPH Oxidase Homolog *NOH-1*. *Science* 287:138, 2000.
4. Geiszt, M., Kopp, J.B., Varnai, P. and Leto, T.L. Identification of Renox, an NAD(P)H Oxidase in Kidney. *Proc. Natl. Acad. Sci USA* 97:8010, 2000.
5. Shiose, A., Kuroda, J., Tsuraya, K., Hirai, M., Hirakata, H., Naito, S., Hattori, M., Sakaki, Y., and Sumimoto, H. A Novel Superoxide-producing NAD(P)H Oxidase in Kidney. *J. Biol. Chem.* 276:1417, 2001.